

# Role of host tissues for sustained humoral effects after endothelial progenitor cell transplantation into the ischemic heart

Hyun-Jai Cho, Namho Lee, Ji Yoon Lee, Yong Jin Choi, Masaaki Ii, Andrea Wecker, Jin-Ok Jeong, Cynthia Curry, Gangian Qin, and Young-sup Yoon

Division of Cardiovascular Research, Caritas St. Elizabeth's Medical Center, Tufts University School of Medicine, Boston, MA 02135

Noncellular differentiation effects have emerged as important mechanisms mediating therapeutic effects of stem or progenitor cell transplantation. Here, we investigated the expression patterns and sources of humoral factors and their regional and systemic biological effects after bone marrow (BM)-derived endothelial progenitor cell (EPC) transplantation into ischemic myocardium. Although most of the transplanted EPCs disappeared within a week, up-regulation of multiple humoral factors was sustained for longer than two weeks, which correlated well with the recovery of cardiac function. To determine the source of the humoral factors, we injected human EPCs into immunodeficient mice. Whereas the expression of human EPC (donor)-derived cytokines rapidly decreased to a nondetectable level within a week, up-regulation of mouse (recipient)-derived cytokines, including factors that could mobilize BM cells, was sustained. Histologically, we observed higher capillary density, a higher proliferation of myocardial cells, a lower cardiomyocyte apoptosis, and reduced infarct size. Furthermore, after EPC transplantation, BM-derived stem or progenitor cells were increased in the peripheral circulation and incorporated into the site of neovascularization and myocardial repair. These data indicate that myocardial EPC transplantation induces humoral effects, which are sustained by host tissues and play a crucial role in repairing myocardial injury.

## CORRESPONDENCE

Young-sup Yoon:  
young.yoon@tufts.edu

Abbreviations used: Ang, angiotensin; BM-MNC, BM-derived mononuclear cell; BMT, BM transplantation; EC, endothelial cell; eGFP, enhanced GFP; EPC, endothelial progenitor cell; FGF, fibroblast growth factor; FISH, fluorescent in situ hybridization; HGF, hepatocyte growth factor; HSC, hematopoietic stem cell; IGF, insulin-like growth factor; IHC, immunohistochemistry; LDL, low density lipoprotein; LV, left ventricle; MI, myocardial infarction; PDGF, platelet-derived growth factor; PIGF, placenta growth factor; qRT-PCR, quantitative real-time RT-PCR; SDF, stromal cell-derived factor; TUNEL, Tdt-mediated dUTP-biotin nick-end labeling; VEGF, vascular endothelial growth factor.

Ischemic heart disease is a worldwide health problem and, in consequence, heart failure has emerged as a leading cause of morbidity and mortality (1). Myocardial infarction (MI) and ischemic cardiomyopathy are typified by the irreversible loss of cardiomyocytes and vasculature, which are essential for maintaining cardiac integrity and function. The recent identification of stem and progenitor cells has triggered attempts to directly regenerate or repair these tissues by cell transplantation. Provocative and hopeful reports describing examples of cardiac regeneration with BM-derived stem (2–4) and progenitor cells (5, 6) have furthered enthusiasm for the use of these cells, yet

many questions remain regarding their therapeutic potential and the mechanisms responsible for these therapeutic effects. In earlier studies, the transdifferentiation of stem or progenitor cells into cardiomyocytes or endothelial cells (ECs) had been proposed as the primary mechanism underlying the therapeutic benefits of cell transplantation (2, 3, 5, 7). However, recent experimental studies using transgenic animal models and imaging techniques have raised questions regarding the magnitude or presence of the transdifferentiation of BM-derived hematopoietic stem or progenitor cells (8–10). Acknowledging that there are differences with regard to study designs, technical differences, and types of transplanted cells used among different studies, the notion that durable engraftment and transdifferentiation of transplanted cells are primarily responsible for therapeutic effects is now being challenged (11–14).

Meanwhile, a paracrine mechanism has been proposed as an additional or alternative mechanism

H.-J. Cho and N. Lee contributed equally to this work.

H.-J. Cho's present address is Dept. of Internal Medicine, Seoul National University Hospital, Yongon-Dong, Seoul 110-744, South Korea.

N. Lee's present address is Div. of Cardiology, Kangnam Sacred Heart Hospital/Hallym University School of Medicine, Seoul 150-950, South Korea.

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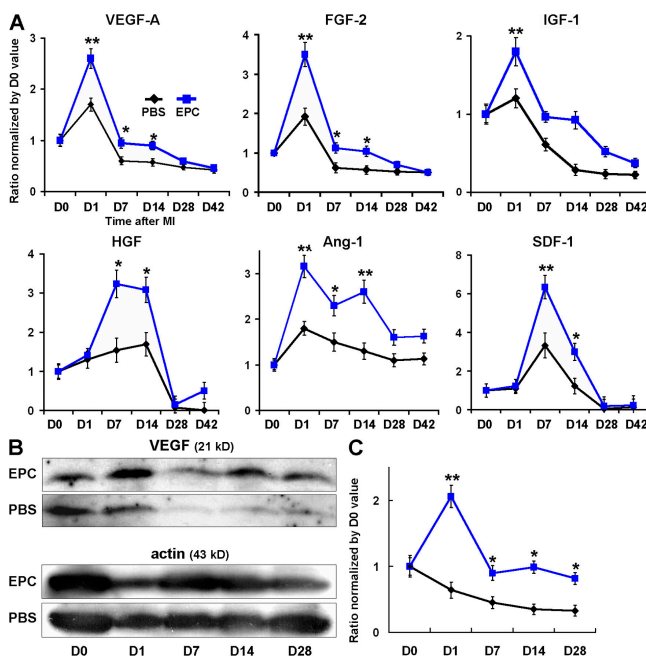
for the therapeutic effects of stem or progenitor cells in ischemic cardiovascular disease (15). In contrast to the hype of pre-clinical and early clinical studies of cell therapy in MI, little is known about the expression of paracrine factors and the resultant effects *in vivo* after cell transplantation in this condition. Although Gnecci et al. (16) investigated this paracrine mechanism, they used Akt-transfected mesenchymal stem cells and did not directly measure paracrine factors. In addition, other studies investigating the paracrine effects of endothelial progenitor cells (EPCs) or mononuclear cells have been limited to *in vitro* assays and/or a hindlimb ischemia model (15, 17, 18). Because the paracrine effects of transplanted cells *in vivo* are variable according to the type of administered cells, the type of disease models, the route and timing of cell administration, etc., we determined to investigate these effects with the use of EPCs in a setting of acute MI, which is one of the most common candidates for cell therapy in the cardiovascular field. In this study, we used the term “humoral” rather than “paracrine” to broadly cover the secretory aspects of cells because autocrine interaction among transplanted cells or systemic action of transplanted cells could also confound these effects. We selected BM-derived EPCs for this study because EPCs are prototype progenitor cells and one of the most widely investigated cell types. EPCs are characterized by sharing hematopoietic stem cell (HSC) markers, such as *c-kit* and *Sca-1* in mice and CD34 and CD133 in humans, but distinctly expressing vascular endothelial growth factor (VEGF)R-2 (19–21). Prior studies demonstrated that culture-expanded EPCs were therapeutically effective in repairing limb ischemia and MI, and incorporation and transdifferentiation of transplanted EPCs into the neovasculature were considered major mechanisms underlying the therapeutic effects (7, 22).

Accordingly, we sought to investigate whether multiple humoral factors are modulated after EPC transplantation in an MI model, and whether these factors are responsible for certain therapeutic mechanisms that are difficult to explain by transdifferentiation of EPCs. We focused on examining representative humoral factors, the duration of their expression in myocardium, the source of these factors, and the resultant biological effects at the cellular level. To meet this end, we adopted certain novel approaches. For example, we transplanted human EPCs into athymic mice to identify the source of cytokines. We also used a mouse BM transplantation (BMT) model in which BM was reconstituted with GFP-expressing cells to track the fate of BM-derived cells in the infarcted myocardium after myocardial EPC transplantation. In this study, we found that myocardial transplantation of EPCs augmented various humoral factors involved in angiogenesis, antiapoptosis, and chemoattraction of BM cells. Intriguingly, these factors were highly expressed in myocardium even after the point at which most of the transplanted cells had disappeared, and up-regulation in this phase was due to recipient cells. Finally, these humoral effects promoted proliferation of host myocardial cells, attenuated apoptosis of jeopardized cardiomyocytes, and augmented homing of BM-derived stem and/or progenitor cells into the ischemic myocardium, which provided a favorable milieu for ongoing neovascularization.

## RESULTS

### Myocardial EPC transplantation induces prolonged expression of multiple humoral factors

To determine the levels of expression of various cytokines in the myocardium after EPC transplantation, we induced MI in C57BL/6 mice by coronary ligation and transplanted  $5 \times 10^5$  cultured mouse EPCs directly into the ventricular wall of the peri-infarct area. PBS was injected as a control. Mice were killed, and cardiac tissues were harvested from the peri-infarct border zone at baseline (before MI, day 0) and 1, 7, 14, 28, and 42 d after treatment. We measured mRNA levels of multiple angiogenic, antiapoptotic, and chemoattractant cytokines in myocardium using quantitative real-time RT-PCR (qRT-PCR). The patterns of expression varied according to each factor and time (Fig. 1 A). At day 1, MI itself (PBS injection) induced an overall increase in cytokine expression, and EPC transplantation significantly augmented such response. At day 7, although the expression levels of VEGF-A, fibroblast growth factor (FGF)-2 (bFGF), and insulin-like growth factor (IGF)-1 fell below baseline levels in the PBS group, baseline levels were maintained in the EPC-transplanted group. Hepatocyte growth factor (HGF) and angiopoietin (Ang)-1 levels increased mildly over 14 d

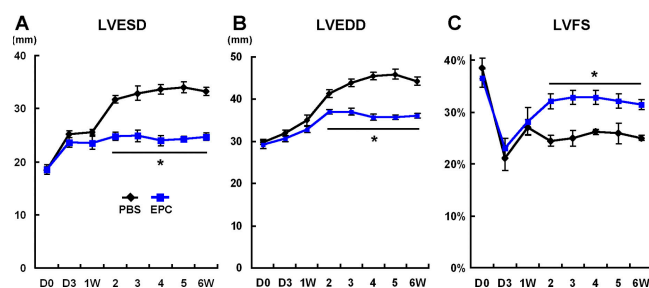


**Figure 1. Temporal expression patterns of multiple cytokines in peri-infarct myocardium after EPC transplantation.** (A) qRT-PCR was performed to measure the level of gene expression from the samples harvested from the peri-infarct myocardium of C57BL mice at baseline (before MI, day 0), 1, 7, 14, 28, and 42 d after MI. Compared with the PBS-injected hearts, hearts transplanted with syngeneic mouse EPCs expressed higher levels of measured cytokines during the study period. Of note, cytokine levels were maintained at, or higher than, baseline levels for at least 14 d only in the EPC-transplanted hearts ( $n = 5$  per group). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . (B and C) Immunoblots (B) and their quantification (C) showed that the protein levels of VEGF correlated well with the mRNA expression levels ( $n = 3$  per group). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

after MI in the PBS group, but they went up to supraphysiologic levels in the EPC group. Uniquely, the level of stromal cell–derived factor (SDF)–1 expression after MI significantly increased until day 7 in the PBS group compared with the baseline level, and EPC transplantation further increased SDF-1 levels. Overall, all the measured cytokines were more highly expressed in the EPC-transplanted hearts compared with the PBS-injected hearts for at least 14 d. Although the expression levels of various cytokines reached their nadir between 7 and 14 d in the PBS group, the changes continued for 14 d and reached their nadir between 28 and 42 d in the EPC group. These data suggest that EPC transplantation not only augments an initial surge of cytokines after MI, but exerts prolonged humoral effects. Next, to verify the correlation between the mRNA and protein levels, we serially measured protein expression of one representative humoral factor, VEGF, by Western blotting. Immunoblots showed that the levels of VEGF were consistently higher in the EPC group compared with the PBS group over 28 d, and the protein levels correlated well with the mRNA levels (Fig. 1, B and C).

#### Therapeutic effects of EPC transplantation occur over the first 2 wk

To investigate the temporal relationship between these cytokines and cardiac function, we performed echocardiography every week for 6 wk after MI and treatment (Fig. 2). Echocardiography showed that from 2 wk after treatment, left ventricle (LV) dimensions at both systole and diastole were significantly smaller in the EPC group than in the PBS group ( $n = 7$  in each group) (Fig. 2, A and B), and LV fractional shortening was better in the EPC group accordingly (Fig. 2 C). These findings suggest that EPC transplantation attenuates adverse ventricular remodeling within 2 wk after MI and prevents cardiac dysfunction in the long run. Furthermore, the time course of cardiac functional improvement correlated well with the temporal expression patterns of the measured cytokines. Therefore, in the following series of experiments, we determined to focus on the molecular and histopathologic changes over the first 2 wk.



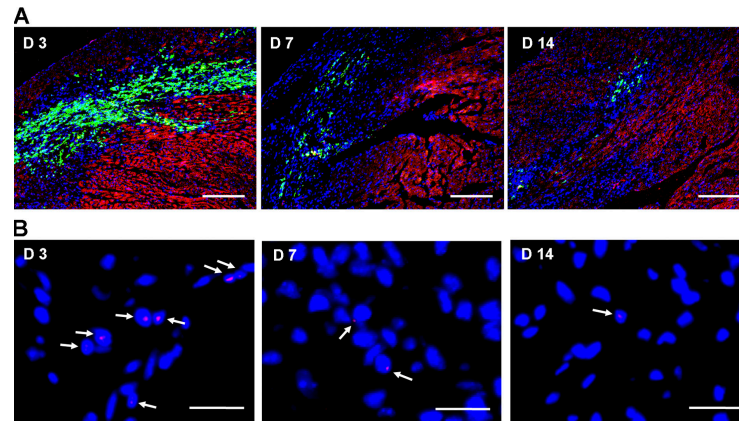
**Figure 2. Therapeutic effects of EPC transplantation occur within the first 2 wk.** Cardiac function measured by M-mode echocardiography was better in EPC-transplanted hearts compared with the control from 2 wk after MI represented by smaller LV end systolic dimension (LVESD) (A) and LV end-diastolic dimension (LVEDD) (B), and greater fractional shortening (C) ( $n = 7$  per group). \*,  $P < 0.05$ .

#### Engrafted EPCs fade away within a week after transplantation

To investigate engraftment characteristics of transplanted cells in the infarcted myocardium, we injected cells immediately after MI, when the engraftment and inflammatory signals are stronger. Because studies have demonstrated that more cells are retained within the myocardium after intramyocardial versus intravenous injection, we selected intramyocardial delivery for this study (11, 23). To track the engrafted cells, EPCs from enhanced GFP (eGFP) mice with a C57BL/6 background were transplanted into wild-type syngeneic mice. Cardiac tissues were harvested at days 3, 7, and 14 after cell transplantation and were subjected to immunohistochemistry (IHC) ( $n = 4$ , at each time point) (Fig. 3 A). In the peri-infarct border zone, we observed robust engraftment of EPCs 3 d after transplantation, which faded away over 7–14 d. Next, to further confirm the disappearance of engrafted cells using a more specific method, we performed fluorescent in situ hybridization (FISH) using a Y chromosome probe. For these experiments, we transplanted male EPCs into female mice and harvested cardiac samples. FISH revealed that the number of Y chromosome signals were significantly higher at day 3 and gradually decreased over 14 d, further confirming the results of IHC (Fig. 3 B). We also explored the potential of EPCs for transdifferentiation. At day 14, a small fraction of EPCs expressed an endothelial phenotype (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20070166/DC1>). Intriguingly, qRT-PCR data revealed that humoral activities were sustained >14 d. Collectively, these data suggest that, even without having many remaining engrafted cells, humoral activities can be sustained. This discrepancy led us to further investigate the role of humoral factors in mediating therapeutic effects of cell transplantation.

#### Multiple angiogenic and antiapoptotic factors are up-regulated after EPC transplantation

First, to confirm and extend our cytokine studies, we determined to measure wider varieties of cytokines at both mRNA and protein levels and included an EC-transplanted group as another control to rule out the nonspecific effects of cell transplantation. We induced MI in mice and randomly assigned them into an EPC, EC, or PBS group ( $n = 5$ , each group). Surgeries and treatment were conducted in an identical fashion to the above experiments. Mice were killed at 2 wk, and cardiac tissues were harvested for the following studies. qRT-PCR showed that various angiogenic factors such as VEGF-A and FGF-2 were significantly up-regulated in the EPC transplantation group compared with the PBS- and EC-treated groups (Fig. 4 A). Other angiogenic factors such as Ang-1, Ang-2, placenta growth factor (PlGF), and HGF were also significantly up-regulated. Interestingly, Ang-2 was increased by 4.4-fold, whereas Ang-1 increased by 2.0-fold in the EPC group compared with the PBS group. We noted relative predominance of Ang-2 to Ang-1, i.e., reversal of Ang ratios in favor of Ang-2 in EPC-transplanted hearts, which were reported to be an evidence of a proangiogenic microenvironment (24, 25). Furthermore, antiapoptotic and cardiomyogenic factor IGF-1 was significantly up-regulated.



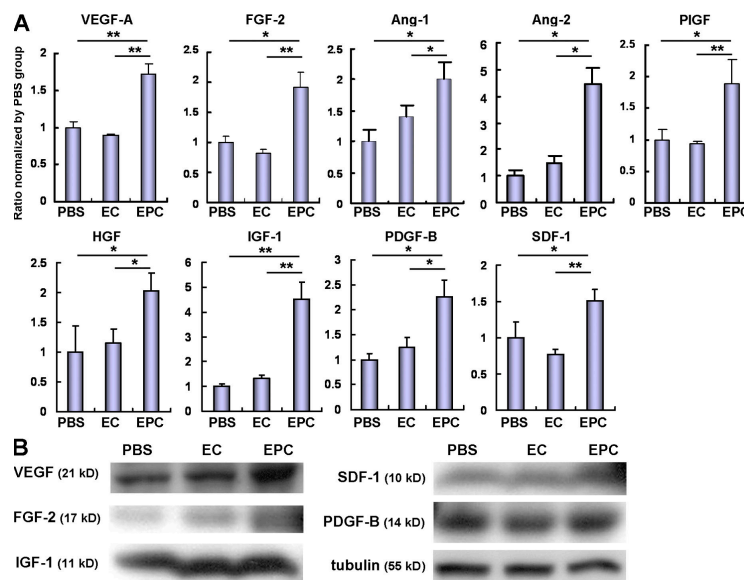
**Figure 3. Engrafted EPCs faded away within a week after transplantation.** (A) EPCs isolated from GFP-expressing mice were injected directly into the myocardium of wild-type mice. Shown are the histological sections of myocardium harvested at predetermined time points. Despite the robust initial engraftment of transplanted EPCs (green) at day 3, a majority of transplanted cells faded away within a week, and only a few transplanted cells were detected in the peri-infarct area at days 7 and 14. green, GFP; red, anti- $\alpha$ -sarcomeric actinin; blue, DAPI. (B) Representative figures from serial cardiac samples (days 3, 7, and 14) obtained from female mouse hearts transplanted with male mouse EPCs (wild-type). The panels show a gradual decrease of transplanted EPCs over 14 d. Red fluorescence within DAPI<sup>+</sup> nuclei (blue) represents Y chromosome signals detected by FISH. Bars: A, 200  $\mu$ m; B, 20  $\mu$ m.

An arteriogenic factor, platelet-derived growth factor (PDGF)-B, and a well known chemoattractant factor, SDF-1, were also up-regulated. Next, we performed immunoblots to examine the protein expression of representative factors such as VEGF, FGF-2, IGF-1, PDGF-B, and SDF-1 in the same cardiac tissues (Fig. 4 B). The levels of these proteins were also elevated in the EPC group compared with the control groups, indicating good correlation with mRNA expression levels. Collectively, these findings indicate that EPC transplantation up-regulates the expression

of multiple humoral factors associated with angiogenesis, anti-apoptosis, cell proliferation, and BM mobilization both at the mRNA and protein levels for at least 2 wk.

#### Sustained up-regulation of humoral factors is attributed to host tissues

From these repeated experiments, it seems clear that these representative cytokines, which are known to be beneficial for cardiac repair, were up-regulated despite scant cellular



**Figure 4. Multiple humoral factors are up-regulated after EPC transplantation.** (A) qRT-PCR using tissues obtained from the peri-infarct myocardium of wild-type mice at day 14 after treatment demonstrated that various angiogenic, antiapoptotic, and chemoattractant cytokines were up-regulated in the mouse EPC-transplanted hearts compared with the PBS- or mouse EC-injected hearts. Individual values were normalized to GAPDH. Data are presented as fold difference compared with the PBS group ( $n = 8$  per group). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . (B) Immunoblots showed that representative humoral factors were also up-regulated at the protein level. Protein expression correlated well with mRNA expression. Blots represented at least four independent experiments.

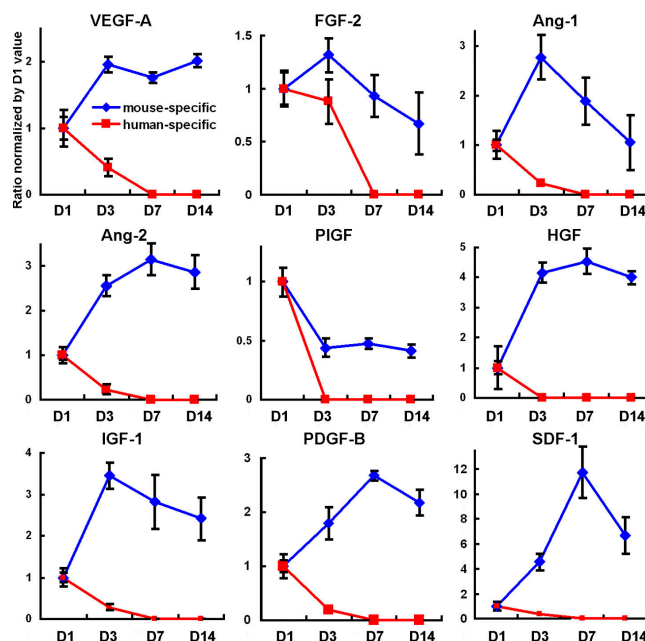


engraftment at 2 wk. Thus, our next step was to explore the source of the increased cytokines. We hypothesized that this prolonged increase in biological factors may be due to host cells. To distinguish between gene up-regulation by donor cells as opposed to recipient cells, we transplanted human EPCs into the myocardium of athymic nude mice. The surgical and cell transplantation procedures were identical to those described in the experiments with mouse EPCs. To detect the origin of cytokines, human- and mouse-specific probes and primers were designed for each humoral factor, and the specificity was tested using cultured human EPCs and mouse heart tissues. qRT-PCR showed no cross-reaction between species-specific primers and probes for the same cytokines (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20070166/DC1>). Considering that the inflammatory response of immunocompromised mice (nude mice) might be different from that of wild-type mice, we performed another preliminary experiment to verify whether the expression levels of cytokines in human EPC-transplanted nude mice are similar to those in mouse to mouse experiments. Similar to the experiments conducted in Fig. 4, we compared the levels of cytokines between human EPC- and control (PBS)-injected hearts in nude mice by qRT-PCR. The human EPC-injected hearts showed robust up-regulation of FGF-2, Ang-2, HGF, IGF-I, and SDF-1, and modest up-regulation of VEGF-A, Ang-1, PlGF, and PDGF-B compared with PBS-injected hearts (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20070166/DC1>). The overall pattern of expression in immunocompromised mice after human EPC transplantation was similar to the results obtained from mouse hearts injected with mouse EPCs (Fig. S2 and Fig. 4 A).

Next, we serially measured the cytokines with both human- and mouse-specific primers and probes. On the whole, the cytokine expressions originating from human EPCs (donor cells) peaked at day 1 and declined to an undetectable range within 7 d, whereas those derived from the mice hearts (recipient cells) went up after day 1 and were maintained over 14 d (Fig. 5). Collectively, these findings suggest that the initial up-regulation is derived from a combination of transplanted and recipient cells, and the sustained up-regulation is attributed to recipient cells, but not to transplanted cells.

#### EPC transplantation decreases apoptosis and augments proliferation of myocardial cells

Next, we examined the effects of increased humoral factors at the cell and tissue levels. Because biological factors associated with cell survival and antiapoptosis such as VEGF, HGF, and IGF-1 were up-regulated, we first investigated whether transplanted cells could affect apoptosis, generally regarded as one of the major mechanisms responsible for ongoing myocardial degeneration after acute MI. We performed a Tdt-mediated dUTP-biotin nick-end labeling (TUNEL) assay with tissue sections harvested from the peri-infarct zone at day 7 (Fig. 6, A and B). The number of TUNEL<sup>+</sup> nuclei in the peri-infarct zone was almost three times lower in the EPC group than in the control groups (EPC group vs. EC group and PBS group:  $19.9 \pm 4.9/\text{mm}^2$  vs.  $57.1 \pm 10.4/\text{mm}^2$  and  $66.6 \pm 16.3/\text{mm}^2$ ;

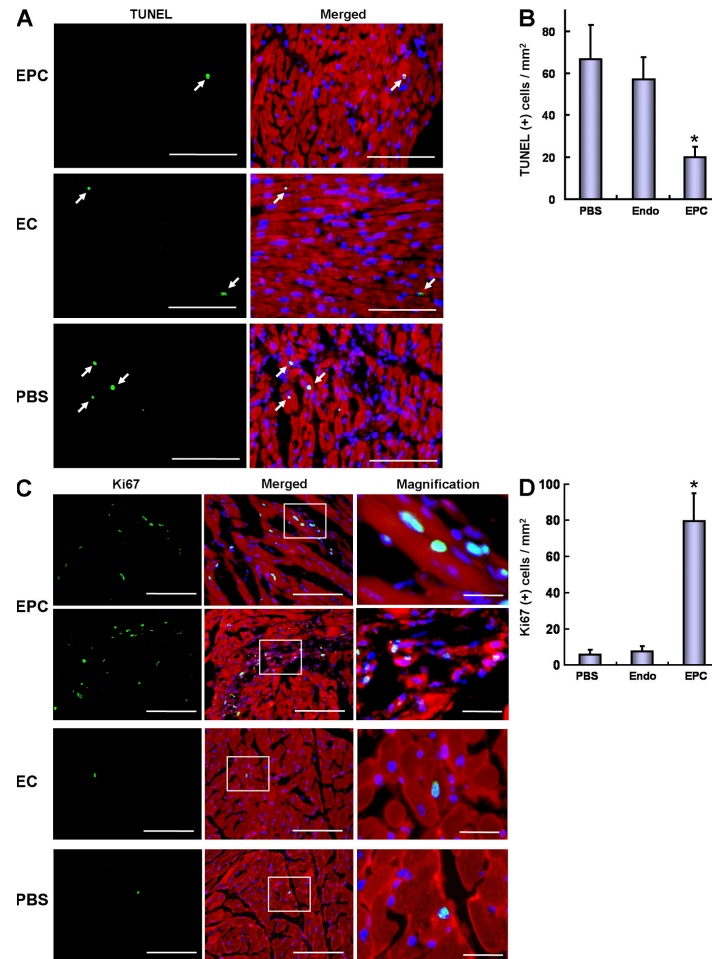


**Figure 5. Host cells constitute the major role for sustained up-regulation of the humoral factors.** To determine whether the up-regulated cytokines were derived from injected donor cells or recipient host cells, human EPCs were transplanted into immunocompromised nude mice, and the levels of cytokines were measured by both human- and mouse-specific primers and probes for each cytokine. The expression levels of cytokines from human EPCs (donor cells) were at their highest levels at day 1 and fell to an undetectable range within 7 d. Most of the mouse (host)-specific cytokine levels continued to rise after day 1 and were maintained at higher than the baseline levels over 14 d ( $n = 5$  per each time point). Individual values were normalized to GAPDH and shown as fold difference to the values at day 1.

$P < 0.01$ ). Furthermore, we studied the pro-proliferative effects of EPC transplantation because cytokines related to cardiomyocyte protection and proliferation such as IGF-1 and PDGF-B were up-regulated. From tissue sections obtained at day 14, we counted cells stained positive for Ki-67. Ki-67 is known to be expressed in all active parts of the cell cycle (G1, S, G2, and mitosis), but not in G0 (26, 27). The number of proliferating cells was 10 times higher in the EPC-transplanted hearts compared with the control groups in the peri-infarct zone (Fig. 6, C and D) (EPC group vs. EC group and PBS group:  $79.7 \pm 15.3/\text{mm}^2$  vs.  $7.55 \pm 2.7/\text{mm}^2$  and  $5.66 \pm 2.7/\text{mm}^2$ ;  $P < 0.01$ ). In the EPC group, we observed small, elongated  $\alpha$ -sarcomeric actinin<sup>+</sup> proliferating cells, suggestive of regenerating immature cardiomyocytes originating from resident cardiac stem or progenitor cells (Fig. 6 C, EPC, bottom).

#### Intramyocardial transplantation of EPCs enhances mobilization of endogenous EPCs and HSCs

Our finding that EPC transplantation substantially increases myocardial expression of VEGF and SDF-1, which are known to mobilize HSCs or EPCs from BM (28, 29), prompted us to investigate the effects of intramyocardial EPC transplantation



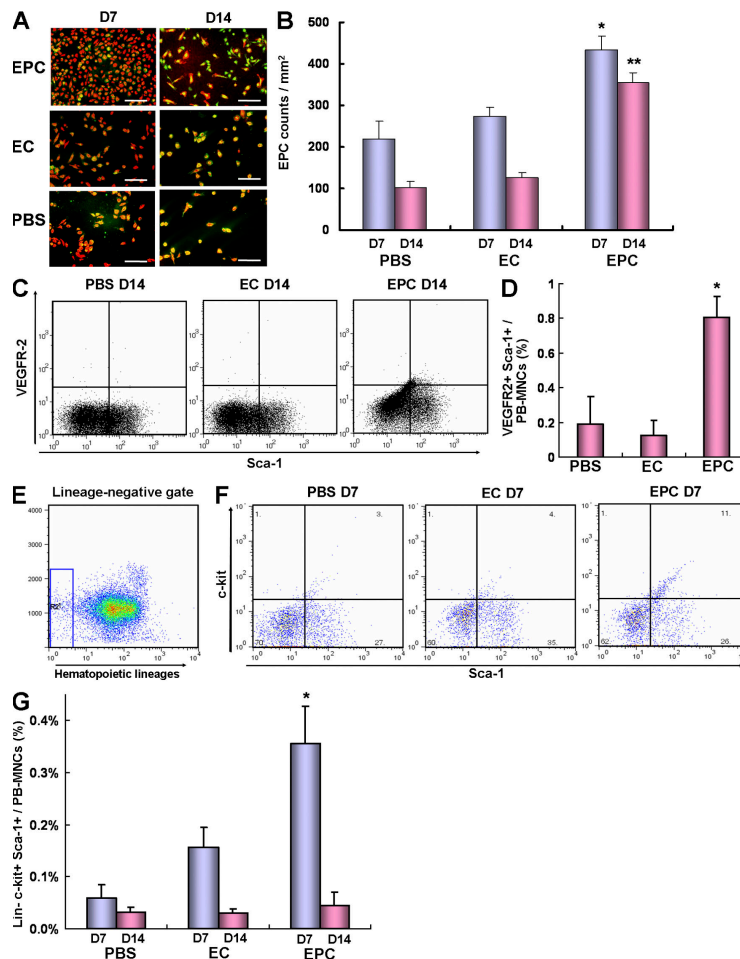
**Figure 6. EPC transplantation decreases myocardial apoptosis and augments proliferation of myocardial cells.** (A and B) Concomitant staining of EPC-, EC-, and PBS-treated myocardium with  $\alpha$ -sarcomeric actinin (red) and TUNEL (green) to identify apoptotic cardiomyocytes (arrows) at day 7 after MI (A). Fewer apoptotic cells were evident in the peri-infarct area of mice receiving EPCs compared with those receiving EC or PBS (B) ( $n = 7$  per group). \*,  $P < 0.01$ . (C and D) Identification of proliferating cells by Ki-67 IHC at 14 d after MI. In myocardial sections, Ki-67<sup>+</sup> cells are demonstrated by green fluorescence (C). Proliferating cardiomyocytes with a regular rod shape (EPC, top) and small-sized elongated shape (EPC, bottom) were more frequently observed in the EPC group. Quantification revealed that EPC-transplanted hearts had a significantly higher number of Ki-67<sup>+</sup> myocardial cells compared with the EC- or PBS-injected hearts ( $n = 7$  per group). \*,  $P < 0.01$  versus EC and PBS groups. Bars: A, 100  $\mu$ m; C, left and middle, 100  $\mu$ m; C, right, 20  $\mu$ m.

on mobilization and recruitment of BM-derived cells. First, to determine the number of mobilized EPCs in circulation, we used two independent measurements: EPC culture assay and flow cytometry (FACS) analysis. The EPC culture assay of peripheral blood showed that the number of circulating EPCs, which were defined by uptake of acetylated low density lipoprotein (LDL) and binding to lectin in culture, was 2 and 3.5 times higher in the EPC group compared with control groups at days 7 and 14, respectively (Fig. 7, A and B). Although the number of circulating EPCs increased over 14 d in the EPC group, it decreased rapidly in the EC and PBS groups after day 7. FACS analysis also revealed that the number of EPCs, which are defined by cells coexpressing VEGFR-2 and Sca-1 epitopes, was significantly higher in the EPC group compared with the control groups at day 14 (Fig. 7, C and D). Furthermore, FACS analysis demonstrated that the number of circulating

Lin<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup> cells, which are regarded as multipotent HSCs, was significantly increased in the EPC group compared with the control groups at day 7 (Fig. 7, E–G). Collectively, these data suggest that myocardial EPC transplantation leads to significant mobilization of endogenous EPCs and HSCs into peripheral circulation.

#### EPC transplantation augments recruitment of BM-derived cells into ischemic hearts, which contributes to neovascularization

Because we found that endogenous BM-derived stem and progenitor cells are mobilized after myocardial transplantation of EPCs, we subsequently evaluated the recruitment and incorporation of BM-derived cells into the infarcted myocardium. To track the cells derived from BM, we used a BMT model in which wild-type mice were lethally irradiated and transplanted

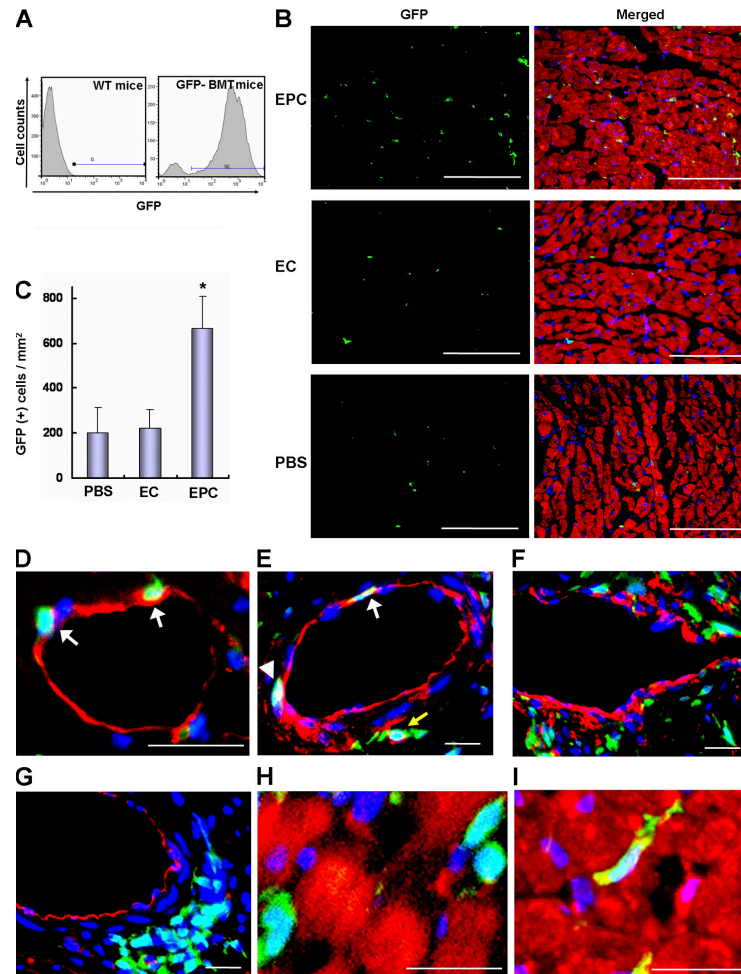


**Figure 7. Intramyocardial transplantation of EPCs enhances mobilization of EPCs and stem cells from BM.** (A and B) EPC culture assay of peripheral blood demonstrated that the number of circulating EPCs, which were defined as adherent cells that showed the characteristics of acetylated LDL uptake (red color) and lectin binding (green color), significantly increased in the EPC group compared with other control groups at days 7 and 14 after MI. \*,  $P < 0.01$  versus EC and PBS at day 7; \*\*,  $P < 0.01$  versus EC and PBS at day 14. (C and D) Flow cytometry analysis showed that the number of double-positive cells for VEGFR-2 and Sca-1 epitopes was significantly increased in the EPC group at day 14. \*,  $P < 0.01$  versus EC and PBS groups. (E–G) Measurement of circulating  $\text{Lin}^- \text{c-kit}^+ \text{Sca-1}^+$  cells by flow cytometry analysis. Hematopoietic lineage $^-$  cells were selected by R2 gate from peripheral blood mononuclear cells after staining with hematopoietic lineage cocktail antibodies (E). Such  $\text{Lin}^-$  cells within the R2 gate were analyzed by c-kit and Sca-1 markers in EPC, EC, and PBS groups (F). Quantitative analysis showed that  $\text{Lin}^- \text{c-kit}^+ \text{Sca-1}^+$  cells in peripheral circulation were significantly increased in the EPC-transplanted group compared with the control groups at day 7 ( $n = 5$  per group). \*,  $P < 0.05$  versus EC and PBS at day 7. Bars: A, 40  $\mu\text{m}$ .

with BM cells of eGFP transgenic mice. 8 wk after BMT, we confirmed reconstitution of BM. FACS analysis showed that  $>90\%$  of peripheral blood mononuclear cells expressed GFP (Fig. 8 A). Then, we performed MI and EPC transplantation in an identical fashion and harvested hearts 2 wk after the surgery. Fluorescent IHC demonstrated that the number of GFP $^+$  cells in cardiac tissues, which indicate cells derived from transplanted BM, was three times higher in the EPC-transplanted group compared with the EC- and PBS-injected groups (Fig. 8, B and C). These data suggest that EPC transplantation not only mobilizes stem and progenitor cells from BM, but also augments recruitment or homing of these BM-derived cells into ischemic myocardium.

Next, to determine the contribution of recruited BM cells to neovascularization and cardiomyogenesis, we performed

fluorescent IHC with vessel and muscle markers. Concomitant staining with GFP- and EC-specific markers such as CD31 (PECAM-1) or isolectin B4 revealed that a portion of GFP $^+$  cells were incorporated into vessels and expressed EC markers, suggestive of transdifferentiation of recruited BM-derived cells into ECs (Fig. 8, D and E, white arrows). Intriguingly, GFP $^+$  cells were more localized in the perivascular and pericytic areas (Fig. 8, F and G). On the other hand, IHC with antibodies against  $\alpha$ -sarcomeric actinin and GFP illustrated that only rarely did recruited BM-derived cells express a cardiomyocyte phenotype, and that these showed the morphology of immature cardiomyocytes, but not mature cardiomyocytes (Fig. 8, H and I). Collectively, these findings suggest that recruited BM-derived cells contribute to neovascularization via direct vasculogenesis as well as potential augmentation of



**Figure 8. EPC transplantation augments incorporation of BM-derived cells into ischemic hearts.** (A) After lethal irradiation, the BM of wild-type mice was reconstituted with BM cells isolated from eGFP transgenic mice. 8 wk after BMT, flow cytometry analysis showed that >90% of peripheral blood mononuclear cells expressed GFP protein, suggesting successful reconstitution of BM. (B) At day 14 after induction of MI in these chimeric mice, the recruited cells from BM to the peri-infarct area were evaluated. Green, GFP; red,  $\alpha$ -sarcomeric actinin; blue, DAPI for nuclei. (C) Quantification of GFP(+) cells showed that EPC transplantation significantly augmented the incorporation of BM-derived cells into the peri-infarct area ( $n = 5$  per group). \*,  $P < 0.01$  versus EC and PBS at day 14. (D–G) IHC with Isolectin B4 (D, red) or CD31 (E–G, red) revealed the localization of BM-derived GFP(+) cells within and around the vasculature in the peri-infarct area at day 14 after MI. White arrows indicate GFP(+) cells, which are both incorporated into vasculature and colocalized with ECs, suggestive of transdifferentiation. A white arrowhead points to the pericytic localization of GFP(+) cells, and a yellow arrow illustrates perivascular localization of GFP(+) cells. The majority of GFP(+) cells were located in the pericytic (F) and perivascular area (G). (H) IHC with  $\alpha$ -sarcomeric actinin (red) revealed that the majority of BM-derived cells did not exhibit a cardiomyocyte phenotype. (I) Rarely, a few GFP(+) cells (arrow) showed immature cardiomyocyte morphology. Bars: B, 100  $\mu$ m; D–I, 20  $\mu$ m.

vascular remodeling or angiogenesis. However, the contribution to direct cardiomyogenesis appears minimal.

#### EPC transplantation decreases infarct size and increases capillary density

Finally, to determine pathological changes after EPC transplantation, we measured fibrosis area and capillary density with 2-wk samples. Picrosirius red staining revealed that fibrosis was significantly reduced in the EPC group (Fig. 9, A and B). Furthermore, isolectin B4 staining showed that capillary density was significantly higher in the EPC group than in the control groups (EPC group vs. EC group and PBS group:

$1,286 \pm 91.9 / \text{mm}^2$  vs.  $742 \pm 98.2 / \text{mm}^2$  and  $559 \pm 92.3 / \text{mm}^2$ ;  $P < 0.01$ ) (Fig. 9, C and D). These results suggest that EPC transplantation increases myocardial neovascularization and induces favorable remodeling of the infarcted heart.

#### DISCUSSION

In this study, we addressed the humoral effects of EPC transplantation in the ischemic heart. Some of the most salient findings of the study are as follows. First, we demonstrated that direct transplantation of BM-derived EPCs into ischemic hearts induces significant and sustained increase in various angiogenic, antiapoptotic, and chemoattractant factors during the



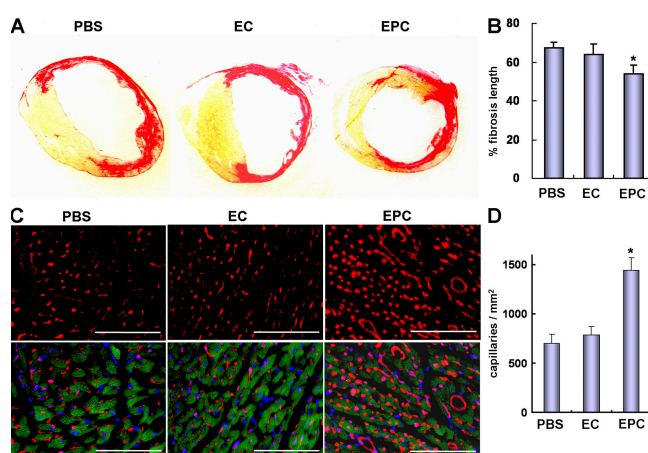
critical period of infarct repair and remodeling. Second, we disclosed for the first time that up-regulation of biological factors after EPC transplantation in ischemic hearts comprises two phases: the initial phase of up-regulation attributed to a combination of transplanted and host cells, and the sustained phase of up-regulation attributed primarily to host cells. Third, myocardial EPC transplantation further mobilizes endogenous BM-derived stem and progenitor cells into peripheral circulation, recruiting them into the ischemic myocardium, and thereby providing an additional favorable milieu for neovascularization and repair or regeneration of ischemic myocardium.

Cell therapy with BM-derived cells for ischemic heart disease has already been introduced into clinical trials (30–32). Although cell therapy has emerged as a promising therapeutic modality, there has been some controversy on the underlying mechanisms. One of the most important debates lies in the plasticity of BM-derived stem or progenitor cells, i.e., whether or not transdifferentiation occurs (2, 3, 7–9, 33). Despite the reported therapeutic benefit of BM-derived stem or progenitor cells, more recent studies demonstrated that the extent and the durability of transdifferentiation are not sufficient to explain the entire therapeutic effects of cell therapy (4, 15–17). In the meantime, a paracrine mechanism has emerged as an alternative or additional mechanism to explain this discrepancy. BM cells are known as a natural source of multiple cytokines involved in angiogenesis. Release of multiple cytokines from BM cellular components, including whole BM-derived mononuclear cells (BM-MNCs) (34), HSCs (35), mesenchymal stem cells (15, 16), and EPCs (17), was demonstrated in previous reports. However, the term “paracrine” seems to be too restricted to encompass the broad secretory components of stem cell effects *in vivo*. For example, cell–cell interaction among transplanted cells via secretory molecules, including

autocrine effects and remote secretory effects of stem cells, are excluded when using the term paracrine. Therefore we adopted the term “humoral” in this study. We assumed that if experiments are designed to study the humoral aspect of cell transplantation, biological factors would need to be measured *in vivo* in a time-dependent manner. Also, the representative biological effects at the cellular and tissue levels need to be investigated together with pathophysiological changes of the heart. In our study, we followed this rationale and demonstrated that multiple angiogenic, antiapoptotic, cardiomyogenic, and chemoattractant factors are significantly increased in EPC-transplanted hearts. Moreover, we first revealed that the time course of the humoral factors correlated well with the functional improvement and favorable cardiac remodeling after MI. Another useful insight was obtained by measuring cytokines at multiple time points. We newly discovered that direct transplantation of EPCs sustains the favorable humoral effects longer than 14 d, which is regarded as the most critical period for determining the fate of cardiac remodeling after acute MI (5, 36).

On the other hand, comparative analysis of data on engraftment and gene expression allowed us to identify the source of up-regulated cytokines, which is one of the most intriguing parts of this study. Classically, the transplanted cells were thought to be a major source of paracrine factors. Previous studies showed that multiple cytokines were increased in the conditioned media of BM-MNC, EPC, and mesenchymal stem cell cultures, and suggested the potential role of released cytokines in mediating therapeutic effects (16, 17, 34). However, no studies broadly addressed the temporal changes, their biological significance, and the role of these factors in the context of myocardial ischemia. In this study, we used a comprehensive approach of physiological, immunohistochemical, and molecular methods to investigate this secretory property of progenitor cells. We noted that, although the majority of transplanted cells had disappeared precipitously within the first week after cell transplantation, most of the beneficial biological factors were still up-regulated 2 wk later. This discrepancy led us to ask if such factors may be derived from host cells. We thus examined the expression levels of the same cytokines with two different sets of primers and probes for each cytokine, designed to detect either human or mouse specific mRNAs, and found that there are dual sources of humoral factors, such that initial up-regulation is caused by transplanted and host cells and sustained up-regulation is due to host cells or tissues.

For the origin of host-derived cytokines after cell transplantation, at least two sources are available: one from resident tissue cells and the other from recruited BM-derived cells. In our model, it is probable that both myocardium and recruited BM cells contribute to the sustained increase of humoral factors. One recent study showed that implanted BM-MNCs in a hindlimb ischemia model did not secrete sufficient angiogenic factors to induce neovascularization, but instead, the stimulated muscle cells produced angiogenic factors (37). That study indicated that the resident muscle cells, but not transplanted cells, could be a major source of angiogenic cytokines.



**Figure 9. EPC transplantation decreases infarct size and increases capillary density.** (A) Representative picosirius red staining of hearts 14 d after MI. The red color represents fibrosis. (B) EPC transplantation significantly reduced percent circumferential fibrosis ( $n = 7$  per group). \*,  $P < 0.05$ . (C) Isolectin B4 staining in the peri-infarct area (red color) at day 14. (D) EPC transplantation significantly increased capillary density ( $n = 7$  per group). \*,  $P < 0.01$  versus EC and PBS groups. Bars: C, 100  $\mu\text{m}$ .

Also, another study recently highlighted the importance of BM-derived stem cells in natural infarct repair (25). This study, with the use of chimeric mice in which BM was reconstituted with c-kit mutant mice and thus BM cell mobilization was defective, demonstrated that this defect of mobilization caused adverse cardiac remodeling after MI and that reconstitution of BM with wild-type BM cells enhanced cardiac repair. Collectively, these studies support the role and importance of resident myocardial cells and mobilized BM cells in the repair and regeneration of tissues after ischemic injury. Here, our study for the first time demonstrated that humoral effects after EPC transplantation provide therapeutic benefits through these host-dependent mechanisms.

Furthermore, we demonstrated the pro-mobilization and -recruitment effects of stem/progenitor cell therapy in this study. Of the multiple cytokines that were increased in the myocardium after EPC transplantation, we noted that VEGF, Ang-1, SDF-1, and PlGF are capable of mobilizing BM cells (38–41). Next, we hypothesized that these increased cytokines could further mobilize BM-derived cells into circulation and facilitate homing of cells into the myocardium. In fact, we found that the number of circulating EPCs and Lin<sup>−</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup> cells in peripheral circulation was increased in the EPC-transplanted animals compared with the animals receiving PBS or EC, and that more BM-derived cells were recruited in the ischemic myocardium. Because these recruited cells are again able to either undergo transdifferentiation or to provide cytokines that are involved in angiomyogenesis and BM mobilization, this mobilization-recruitment-secretion loop appears to play an important role in myocardial repair. Also, we noticed that myocardium serves as a depot for important angiogenic factors. Our data in Fig. 1 revealed that after MI, important angiogenic and antiapoptotic cytokines such as VEGF-A, FGF-2, and IGF-I, which were only mildly elevated due to inflammatory response after acute MI, decreased quickly below the baseline level within 3 d in the border zone due to the loss of cardiomyocytes, suggesting that myocardium itself is an important secretory organ. Therefore, early preservation of myocardial mass is an important target for therapy not only to prevent mechanical dysfunction, but also to maintain proper secretory function. In this context, high expression of IGF-1 and HGF after EPC transplantation may play a crucial role in preventing adverse remodeling, as these two cytokines are known to protect cardiomyocytes from apoptotic cell death and induce proliferation of resident cardiac stem cells for neovascularization and cardiomyogenesis (18, 42).

We also examined the fate of transplanted EPCs (donor) and mobilized BM-derived cells (host-derived cells). Transplanted EPCs have been shown to incorporate into neovasculature in ischemic injury models (6, 7, 22). However, the extent and duration of incorporation of transplanted EPCs into host endothelium varies widely among studies (43). In our observation, the vast majority of transplanted EPCs disappeared within 1 wk, even though we observed robust engraftment at 3 d after cell transplantation. Intriguingly, we confirmed the contribution of mobilized host BM cells to

endothelium at the site of neovascularization even at 2 wk after EPC transplantation. Although the exact colocalization with the endothelium was relatively infrequent, we observed accumulation of more BM-derived cells at the perivascular and pericytic area (Fig. 8). These findings were consistent with the previous report that proposed the essential paracrine role of recruited cells for augmenting neovascularization (44). Collectively, these findings support the importance of pro-mobilization action of transplanted EPCs in repairing ischemic myocardium.

The findings of this study are of interest for a variety of scientific reasons. However, the implication of our data resides to a large degree on the humoral effect of culture-expanded early EPCs in the setting of acute MI. Because definitions of EPCs and candidate diseases differ widely between studies (45, 46), this paradigm alone may not offer the full explanation for the humoral or paracrine effects in other cell therapy studies.

In conclusion, we showed that intramyocardial EPC transplantation induces humoral effects that are sustained by host tissues and play a crucial role in repairing myocardial injury, and we revealed the importance of cross-talk between heart and BM mediated by humoral effects after progenitor cell transplantation for achieving optimal therapeutic effects. Our observation may further extend to the requirement for healthy BM and heart to obtain optimal therapeutic effects after BM-derived progenitor cell therapy in acute MI. This may also partly explain the difference in results between preclinical experiments performed in healthy animals and clinical trials performed in patients with multiple risk factors and diseased myocardium that affect the health of BM and myocardium.

## MATERIALS AND METHODS

### Animals

Wild-type C57BL/6 ubiquitous eGFP-expressing transgenic mice with a C57 background (The Jackson Laboratory) and athymic nude mice (Charles River Laboratories) aged 6–10 wk were used. All experimental protocols were approved by Caritas St. Elizabeth's Institutional Animal Care and Use Committee and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Induction of MI and intramyocardial cell transplantation.** Mice were anesthetized with an intraperitoneal injection of avertin (0.014 mg/kg; 2,2,2-Tribromoethanol; Sigma-Aldrich). Mice were intubated with a 22G IV catheter and artificially ventilated with a mechanical ventilator (Harvard Apparatus). After a left-sided thoracotomy, the left anterior descending artery was ligated just distal to the bifurcation of the diagonal branch using 8–0 polypropylene sutures through a dissecting microscope (47). The apex of the LV was observed for evidence of myocardial blanching and akinesia indicating interruption of coronary flow. After induction of MI,  $5 \times 10^5$  mouse or human EPCs in a volume of 50  $\mu$ l PBS was directly transplanted into the peri-infarct areas and apex (total of five sites) using a 30 G needle. In control groups,  $5 \times 10^5$  strain-matched ECs or the same volume of PBS was injected in an identical fashion. At predetermined time points, peripheral blood samples were obtained and hearts were harvested after mice were killed.

**BMT model.** To trace BM-derived cells, BMT was performed as described previously (48). In brief, each recipient wild-type mouse was lethally irradiated with 1,200 cGy in two equal doses of 600 cGy delivered 3 h apart.  $10^6$  BM-mononuclear cells harvested from eGFP mice in a volume of 200  $\mu$ l PBS were injected into the tail vein.

## Cell cultures

**EPC.** For culture of mouse EPCs, BM-MNCs were isolated by density gradient centrifugation with Histopaque-1083 (Sigma-Aldrich) and were plated at a density of  $0.8\text{--}1.0 \times 10^6$  cells/cm<sup>2</sup> on rat plasma vitronectin-coated (Sigma-Aldrich) dishes with EC basal medium (EBM-2) supplemented with 5% FBS, antibiotics, and cytokine cocktail (SingleQuots; Clonetics). Nonadherent cells were removed at day 4, and cultures were supplemented with new media. The cells were maintained through day 7 and used as EPC-enriched population (7, 22). For cultivation of human EPCs, mononuclear cells were isolated with Histopaque-1077 (Sigma-Aldrich) from 50 ml of peripheral blood obtained from healthy donors and cultured in EPC media as described above. All experiments dealing with human products were conducted with informed consent.

**EC.** Mature ECs of C57BL/6 mice (MS1, pancreatic islet EC line) were purchased (American Type Culture Collection) and cultured in DMEM media supplemented with L-glutamine, 10% FBS, and antibiotics.

## qRT-PCR and immunoblotting

Peri-infarct myocardial tissues were harvested and pulverized to extract RNA or protein. Total RNA was extracted with the use of RNA-Stat (Iso-Tex Diagnostics) according to the manufacturer's instructions. The extracted RNA (500 ng) was subject to cDNA synthesis with Taqman Reverse Transcription Reagents (Applied Biosystems) at a final volume of 20  $\mu$ l. For the PCR reactions of various humoral factors, we used mouse-specific and human-specific primers and probes, respectively (Table S1, available at <http://www.jem.org/cgi/content/full/jem.20070166/DC1>). The number of PCR cycles needed for 6-carboxyfluorescein fluorescence to cross a threshold where a statistically significant increase in change in fluorescence (threshold cycle [CT]) was measured using Lightcycler 3.5 software. Relative RNA expression was determined using the formula  $\text{Rel Exp} = 2^{-\Delta\text{CT}}$ , where  $\Delta\text{CT} = \text{CT gene of interest} - \text{CT GAPDH}$  in experimental samples. To confirm the protein expression of the genes of interest, immunoblot (Western blot) assays were performed by modification of the procedures described previously (4). In brief, protein extracts (100  $\mu$ g per sample) were separated using SDS-PAGE (Bio-Rad Laboratories) and electrotransferred onto PVDF membranes (GE Healthcare). Samples were probed with the following antibodies: VEGF, FGF-2, IGF-I, PDGF-B, and SDF-1 (all from Santa Cruz Biotechnology, Inc). ECL or ECL-PLUS (GE Healthcare) was used for detection. Equal protein loading was confirmed by reprobing with tubulin antibody (EMD).

## Circulating EPC culture assay

EPC culture assay was performed as described previously (38, 49). In brief, peripheral blood mononuclear cells isolated from 500  $\mu$ l of blood by density gradient centrifugation were cultured in the aforementioned EPC media on four-well glass slides. After 4 d in culture, cells were incubated with DiI-labeled acetylated LDL (Biomedical Technologies) for 1 h, followed by FITC-conjugated *Bandeiraea simplicifolia* lectin I (Vector Laboratories). The double-stained cells, considered EPCs, were counted in 10 randomly selected high-power fields under fluorescent microscopy, and the number of positive cells was converted to numbers per square millimeter.

## Flow cytometry cell analysis

Single cell suspensions of peripheral blood mononuclear cells were labeled with antibodies against FITC-conjugated Sca-1 (Ly6AE), PE-conjugated VEGF-R2(LY-73), PE-conjugated c-kit (CD117), and an allophycocyanin-conjugated lineage cocktail (CD3e, CD11b, Ly-6G, GR-1, CD45R/B220, and TER-119; all from BD Biosciences) and analyzed using a FACScan (Becton Dickinson) or a MoFlo flow cytometer (Dako) as described previously (4, 47). Proper isotype-matched IgG was used as a control.

## Physiological assessment of LV function

Transthoracic echocardiography (15.0-MHz ultraband linear transducer; SONOS 5500; Hewlett Packard) was performed before and 2 wk after MI and cell transplantation. LV dimensions in end systole and end diastole as well as fractional shortening were measured as described previously (4, 49).

All measurements represented the mean of at least three consecutive cardiac cycles.

## Histological analysis

Hearts were perfused retrogradely through the right carotid artery with PBS and 4% paraformaldehyde. The hearts were fixed for 4 h in paraformaldehyde and incubated overnight in 15% sucrose solution. The tissues were embedded in OCT compound (Sakura Finetek), snap-frozen in liquid nitrogen, and sectioned at 5-mm thickness as described previously by our laboratory (4, 47). To measure circumferential fibrosis area, picrosirius red staining was performed (50). Percent fibrosis length was calculated as the ratio of the length of fibrosis to the length of LV circumference by using a computerized digital image analysis system (Image Pro, version 4.5; MediaCybernetics). To trace GFP<sup>+</sup> cells in hearts, anti-GFP antibody (Abcam Inc.) was used. To identify capillaries and cardiomyocytes, an EC marker Isolectin B4 (Vector Laboratories) or anti-CD31 antibody (BD Biosciences) and anti- $\alpha$  sarcomeric actinin antibody or anti-troponin-I (Santa Cruz Biotechnology, Inc.) were used. We used DAPI for nuclear counterstaining. To identify male EPCs in the female hearts, FISH was performed with a Cy3-conjugated mouse Y chromosome probe (Cambio). The tissue sections were pre-denatured, dehydrated, denatured, and hybridized according to the manufacturer's protocol. To assess apoptosis, TUNEL reaction was performed by using the fluorescein in situ cell death detection kit (Roche). To evaluate proliferative cells, IHC with anti-Ki-67 antibody (Novocastra Laboratories) was performed. In the above fluorescent IHC, Cy2-, FITC-, Cy3-, rhodamine-, or TRITC-conjugated secondary antibodies were applied appropriately. For quantification of apoptosis, capillary densities, and proliferating cells, at least four randomly selected fields at both sides of the peri-infarct border zone in nonconsecutive tissue sections were examined, and the number of positive cells in each high-power field was converted to cells per square millimeter.

## Statistical analysis

All data were presented as mean  $\pm$  SE. Statistical analyses were performed with Student's *t* test for comparisons between two groups, and ANOVA followed by Bonferroni's correction was performed for more than two groups using SPSS version 11.0. *P* < 0.05 is considered to denote statistical significance.

## Online supplemental material

Table S1 describes the mouse-specific and human-specific primers and probes for qRT-PCR. Fig. S1 shows the specificity of these primers and probes. Fig. S2 describes the expression patterns of humoral factors in the MI hearts of athymic nude mice 14 d after human EPC transplantation. The online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20070166/DC1>.

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